

Sodium dichloroacetate selectively targets cells with defects in the mitochondrial ETC

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The “Warburg effect,” also termed aerobic glycolysis, describes the increased reliance of cancer cells on glycolysis for ATP production, even in the presence of oxygen. Consequently, there is continued interest in inhibitors of glycolysis as cancer therapeutics. One example is dichloroacetate (DCA), a pyruvate mimetic that stimulates oxidative phosphorylation through inhibition of pyruvate dehydrogenase kinase. In this study, the mechanistic basis for DCA anti-cancer activity was re-evaluated *in vitro* using biochemical, cellular and proteomic approaches. Results demonstrated that DCA is relatively inactive (IC₅₀ ≥ 17 mM, 48 hr), induces apoptosis only at high concentrations (≥25 mM, 48 hr) and is not cancer cell selective. Subsequent 2D-PAGE proteomic analysis confirmed DCA-induced growth suppression without apoptosis induction. Furthermore, DCA depolarizes mitochondria and promotes reactive oxygen species (ROS) generation in all cell types. However, DCA was found to have selective activity against rho(0) cells [mitochondrial DNA (mtDNA) deficient] and to synergize with 2-deoxyglucose in complex IV deficient HCT116 p53(-/-) cells. DCA also synergized *in vitro* with cisplatin and topotecan, two antineoplastic agents known to damage mitochondrial DNA. These data suggest that in cells “hardwired” to selectively utilize glycolysis for ATP generation (*e.g.*, through mtDNA mutations), the ability of DCA to force oxidative phosphorylation confers selective toxicity. In conclusion, although we provide a mechanism distinct from that reported previously, the ability of DCA to target cell lines with defects in the electron transport chain and to synergize with existing chemotherapeutics supports further preclinical development.

The Warburg effect, also known as aerobic glycolysis, describes the shift in ATP generation in cancer cells from oxidative phosphorylation to the less efficient process of glycolysis, even in the presence of oxygen.¹⁻³ The ability of cancer cells to

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Additional Supporting Information may be found in the online version of this article.

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“hardwire” aerobic glycolysis has several advantages including activation of pro-survival pathways, increased lactic acid production and preservation of acetyl-CoA as a carbon source.^{4,5} It is thought selective pressure within hypoxic tumor microenvironments drives the evolution of clones with a glycolytic phenotype.⁶ Several molecular mechanisms have been proposed to account for this shift. First, sequencing of mitochondrial DNA (mtDNA) from tumors reveals widespread loss of function mutations in respiratory chain components.⁷ This physical block requires that cells increase utilization of glycolysis to maintain ATP generation. Alternatively, the transcription factor hypoxia-inducible factor (HIF-1 α) and oncogenic signaling pathways involving PI3K/AKT/mTor, MYC and RAS have been shown to contribute toward a glycolytic shift.^{2,8-10} Likewise, loss of the tumor suppressor p53 has similar effects.¹¹ Therefore, it appears the cancer cell can achieve the Warburg transition through multiple overlapping pathways. Irrespective of the mechanisms involved, if certain tumors are reliant on glycolysis for bioenergetics, biosynthesis and invasive potential, therapeutic targeting of the underlying pathways may result in an anti-tumor activity. On this basis, inhibitors of key glycolytic enzymes including hexokinase, phosphofructokinase, GAPDH and lactate dehydrogenase have been studied as potential anti-cancer agents.¹²

A controversial addition to this class of agent is dichloroacetate (DCA), a pyruvate mimetic and inhibitor of pyruvate

dehydrogenase kinase (PDK).^{13,14} The target of DCA inhibition, PDK, acts to reduce pyruvate utilization within the mitochondria through phosphorylation of the E1 α subunit of pyruvate dehydrogenase (PDH). By inhibiting PDK, PDH is then maintained in a catalytically active state, mitochondrial pyruvate consumption is increased, the substrate pool available for glycolysis is lowered and lactic acid production is inhibited.¹³ The ability of DCA to reduce serum lactate levels led to several clinical trials in patients with congenital syndromes where lactic acidosis is a major cause of morbidity (e.g., mitochondrial cytopathies).^{15–18} The consensus from these trials was negligible improvement in clinical outcome with the frequent development of peripheral neuropathy.¹⁷

A recent study by Bonnet *et al.* reported that DCA was also capable of inducing apoptosis in cancerous but not in normal cells *in vitro*.¹⁹ This was combined with significant *in vivo* activity in a human lung cancer (A549) xenograft model in athymic rats. Subsequently, DCA was shown to induce apoptosis in endometrial cancer cells and to sensitize prostate cancer cells to ionizing radiation.^{20,21} Regarding an underlying mechanism, Bonnet *et al.* propose that because cancer cells have hyperpolarized mitochondria (high $\Delta\Psi_m$) with low expression of the potassium transporter Kv1.5, DCA treatment “normalizes” the mitochondrial membrane potential leading to increased H₂O₂ production, efflux of mitochondrial pro-apoptotic factors and activation of plasma membrane Kv1.5 potassium channels, which all conspire to induce cell death. The implications of this study are far reaching, suggesting that maintenance of a hyperpolarized mitochondrial membrane potential is essential for cancer cell survival. Regrettably, the publicity surrounding the initial study, combined with ease of DCA availability, prompted self-medication by patients and even prescription by some physicians.²²

In this study, we undertook a retrospective of DCA *in vitro* activity to verify and extend previously reported mechanistic data.¹⁹ We conclude that (i) DCA is relatively inactive *in vitro* and inhibits cell viability with an IC₅₀ similar to that observed with sodium pyruvate and sodium acetate, (ii) the anti-cancer activity is not selective as DCA displays similar activity toward normal cells, (iii) growth suppression generally occurs without apoptosis induction, (iv) DCA depolarizes mitochondria of both normal and tumor cells, and (v) the potassium transporter Kv1.5 has less overall involvement. However, DCA showed increased activity against cells with mitochondrial defects and effectively synergized with agents known to target mitochondria or interfere with glucose metabolism. These data suggest that clinical evaluation of DCA may benefit from selecting patient populations or combination regimes in accordance with the mechanism described here.

Material and Methods

Materials

Dichloroacetate was obtained from the Drug Synthesis and Chemistry Branch of the Developmental Therapeutics Program, National Cancer Institute (NCI, Rockville, MD); a second reference source was Sigma Chemical Company (St.

Louis, MO). The normal human dermal fibroblasts (NHDF) and human umbilical vein endothelial cells (HUVEC) were from Lonza (Allendale, NJ). A transformed normal fibroblast line (MRC5) cells were from ATCC (Manassas, VA). The rho(0) MOLT4 cell line was provided by Prof. Lionel L. Lewis (Dartmouth-Hitchcock Medical Center, Lebanon, NH). HCT116 p53(–/–) and p21(–/–) cells were provided by Prof. Bert Vogelstein (HHMI, Baltimore, MD). MCF7 rho(0) cells were prepared within our laboratory. All remaining cell lines were from the Division of Cancer Treatment and Diagnosis Tumor Repository (Frederick, MD). Antibodies against cytochrome c, α -enolase, PCNA, survivin and GAPDH were from Abcam (Cambridge, MA). Kv1.5 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), UC Davis NeuroMab Facility and Sigma. Unless otherwise stated, all other reagents and inhibitors were from Sigma.

Cell viability assays

[¹⁴C]-leucine cell viability assays were performed as described earlier.²³ Experiments were performed at least twice with triplicate determinations for each point. The IC₅₀ was defined as the concentration of drug required to inhibit protein synthesis (cell viability) by 50% relative to control-treated cells. For growth measurements in glucose/galactose-containing media, cells in 96-well plates were grown in either glucose-containing media (RPMI-1640 containing 4.5 mg/mL glucose, 0.11 mg/mL pyruvate) or glucose-free media supplemented with galactose (RPMI-1640 containing 0.9 mg/mL galactose and 0.5 mg/mL pyruvate). Both media were supplemented with 10% dialyzed FBS. Cells were incubated for 4 days and growth was determined by incorporation of ¹⁴C-leucine as described above as well as by staining with crystal violet.

Combination studies

Dose response curves of DCA (0.3–30 mM) in combination with either deoxyglucose (0.01–1 mM), rotenone (0.001–1 μ M), cisplatin (0.01–10 μ M), topotecan (0.3–300 nM), wortmannin (1–1,000 nM), or LY294002 (0.03–30 μ M) were assayed at multiple fixed ratios in the cell viability assays as described above. Combination indices (CI) were calculated using CalcuSyn software (Biosoft, Cambridge, UK) using pooled data from at least two experiments with triplicate determinations for each point.

Mitochondrial depolarization assay

Cells grown on coverslips in 6-well dishes were washed once with PBS and 5 mL of serum-containing media were added. Tetramethyl-rhodamine methyl-ester perchlorate (TMRM, Invitrogen), a red fluorescent molecule that accumulates in the mitochondrial matrix, was then added to a final concentration of 0.1 μ M. Following 2 hr loading, the cells were washed with PBS and media supplemented with the appropriate study compound was added. After 2 hr, coverslips were washed, mounted and images acquired using a DM

compound fluorescence microscope (Leica, Rockville, MD). Additional methods can be found in Supporting Information.

Results

DCA is relatively inactive in cell viability assays

A sensitive viability assay based on C^{14} -leucine incorporation was used to calculate the cytotoxicity of DCA across a panel of cell lines. The related compounds monochloroacetate (MCA), sodium pyruvate and sodium acetate were also included as reference agents (Fig. 1a). A representative viability assay for all four compounds in A549 cells is shown in Figure 1b. The IC_{50} values obtained for a panel of cell lines are listed in Figure 1c. Results demonstrate that DCA is relatively inactive in all tumor cell lines, with IC_{50} values ranging from 17 to 40 mM after 48 hr. These values were consistent between cancer cell types, irrespective of histological origin (mean 27 ± 6 mM). Similar results were obtained using DCA from two different sources. Increasing incubation times from 2 to 6 days did not result in any further decrease in viability, suggesting cytostasis (data not shown). Notably, the IC_{50} values for DCA were similar to those obtained with both pyruvate (mean IC_{50} 35 mM \pm 16 mM) and acetate (mean IC_{50} 58 mM \pm 15 mM). Although DCA was relatively inactive as a cytotoxic agent, its activity as a PDK inhibitor was consistent with reports in the literature, *i.e.*, IC_{50} between 10 and 100 μ M (Supporting Information Fig. 1).²⁴ In contrast, MCA, an alkylating agent with well-documented toxicity,^{25,26} was >50 times more active than the other study compounds (mean IC_{50} = 0.4 mM \pm 0.17 mM). As illustrated in Figure 1b, for doses of >30 mM, a rapid decline in cell viability was observed for DCA, pyruvate and acetate, suggesting nonspecific inhibition of cell growth, likely due to hyperosmotic effects. Viability measurements made using a crystal violet assay yielded similar results indicating that the lack of response was not a consequence of assay format (data not shown). Furthermore, IC_{50} values determined for the panel of cell lines were comparable with those obtained for three normal cell types: HUVEC, NHDF and MRC5 (Fig. 1c). Therefore, viability assays suggest that in this panel of cell lines DCA has minimal growth inhibitory activity and is not cancer cell selective.

High-dose DCA is required for apoptosis induction

Apoptosis induction was reported to be a central mechanism by which DCA exerts its effects *in vitro*.^{19–21} To confirm these data, we investigated the effect of increasing concentrations of DCA, MCA, pyruvate and acetate on levels of apoptosis in HL60 cells, a p53 null promyelocytic leukemia line with a high propensity to undergo apoptosis. Results demonstrated that in cells treated with DCA for 48 hr, increased levels of apoptosis were evident only at ≥ 25 mM DCA (Fig. 2a). Similar effects were noted for both pyruvate and acetate. For MCA, significant levels of cell death were seen at all concentrations along with some evidence of late-stage apoptosis induction. Experiments with DCA were then expanded to include additional cell lines: A549, MCF7 and PC3 cells (Fig. 2b). Direct assessment of apoptosis induction showed that, after 48 hr, these lines were largely unresponsive

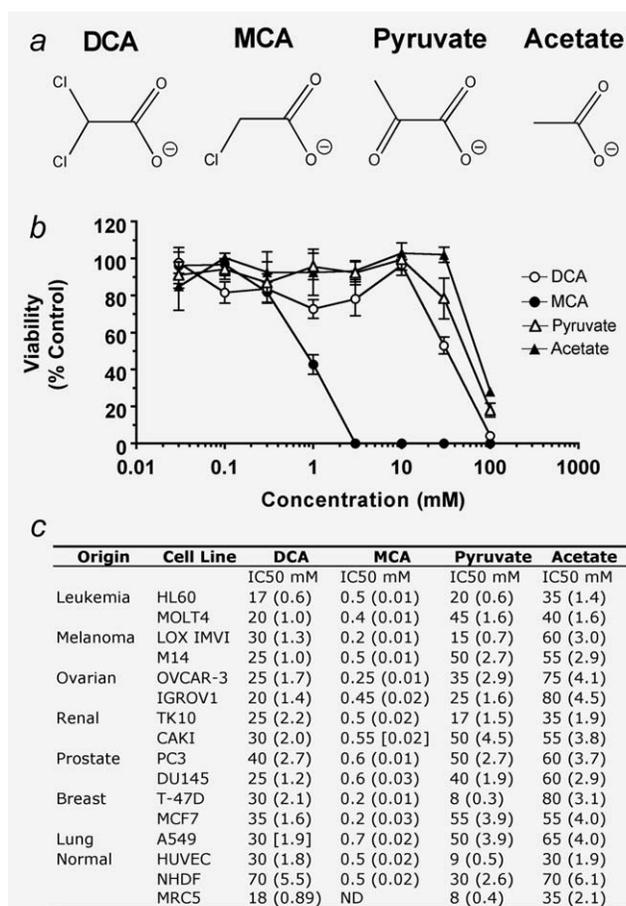


Figure 1. DCA activity relative to structurally related compounds across a panel of cancer and normal cells. (a) Chemical structures for DCA, MCA, sodium pyruvate and sodium acetate. (b) C^{14} leucine viability assay of A549 cells treated with increasing concentrations of DCA, MCA, pyruvate and acetate for 48 hr. (c) Summary of cell viability assays for DCA, MCA, pyruvate, and acetate across a panel of tumor and normal cells. IC_{50} was defined as the concentration of drug required to inhibit [C^{14}]-leucine incorporation by 50% relative to control-treated cells. Assays were performed at least twice with triplicate determinations for each point and the data pooled. Numbers in parentheses are SEM.

even at 50 mM DCA. Subsequent cell cycle analysis confirmed that HL60 cells were the only cells to have a sub- G_0 population, indicative of apoptosis, without phase-specific arrest. MCF7 cells treated with 50 mM DCA showed arrest in G_0 , whereas the other lines did not show phase-specific changes. Next, A549 cells treated with DCA were analyzed by immunocytochemistry for markers of cell proliferation (Fig. 2c). Results demonstrated that at 25 mM DCA, PCNA and survivin reactivity decreased, combined with a significant decline in overall cell number according to DAPI staining. At 25 mM DCA, DAPI staining failed to demonstrate nuclear condensation indicative of apoptosis. As an extension of these assays, A549 cells were treated with 25 mM DCA for 48 hr and analyzed for changes in subcellular localization of cytochrome c (Fig. 2d). Results showed no discernable

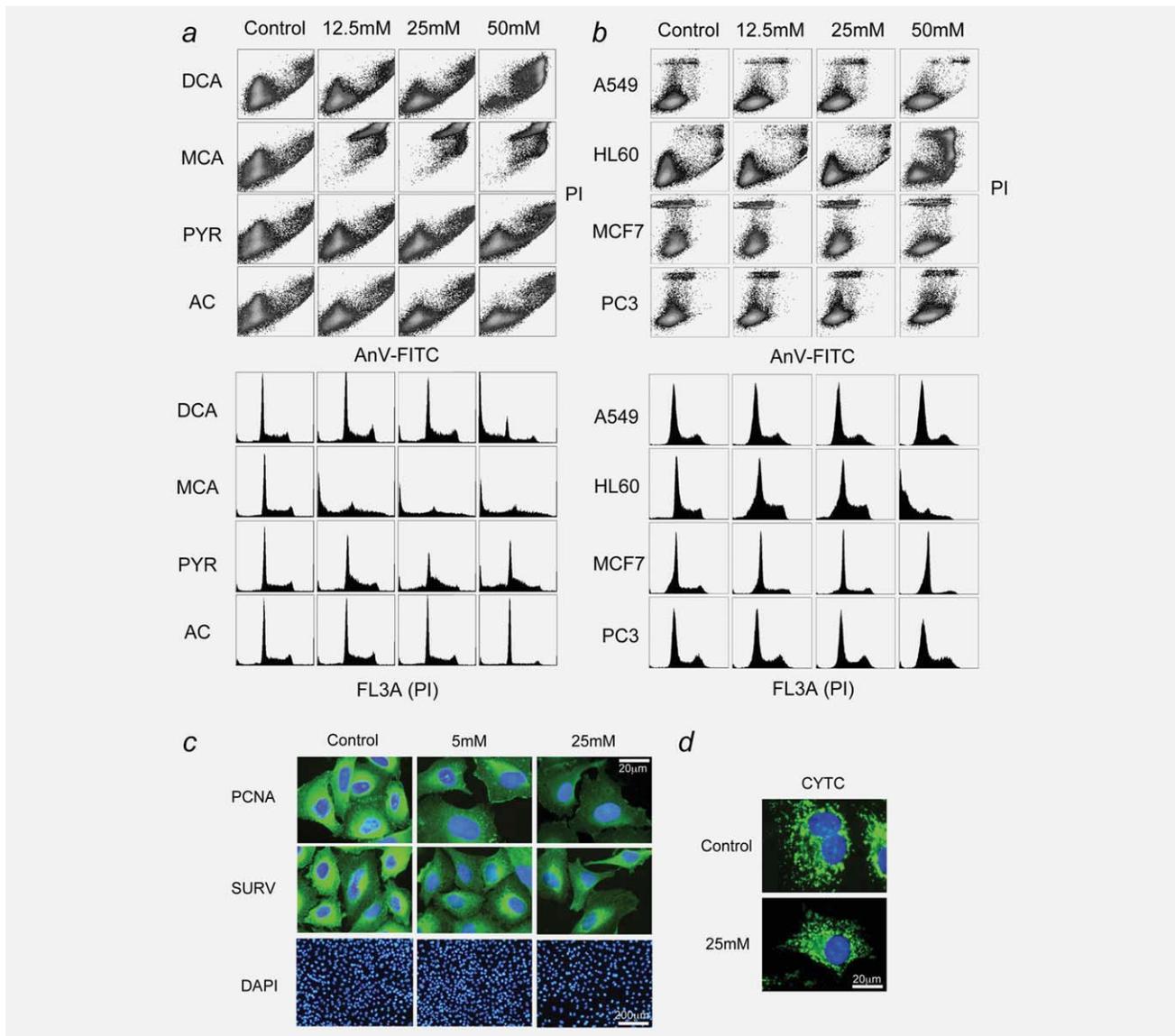


Figure 2. DCA has minimal effect on prevailing levels of apoptosis. (a) HL60 cells were exposed to several concentrations of DCA, MCA, pyruvate (PYR), or acetate (AC) for 48 hr, followed by analysis for apoptosis induction using AnV/PI staining (top panels) or for perturbations in the cell cycle by PI staining (lower panels). (b) A panel of four cell lines was treated with increasing doses of DCA for 48 hr followed by analysis of apoptosis (top panels) and cell cycle alterations (lower panels). Results are representative of three separate experiments. (c) Immunocytochemistry of PCNA/survivin expression and DAPI nuclear distribution for A549 cells treated with DCA for 48 hr (5 and 25 mM). (d) Cytochrome c subcellular localization in A549 cells treated with 25 mM DCA for 48 hr.

change in fluorescence distribution to indicate cytochrome c efflux had occurred. In summary, these data suggest that at clinically relevant doses (<1 mM) DCA does not induce apoptosis. However, at high concentrations (≥ 25 mM) DCA does appear to inhibit cell growth *in vitro*, which is consistent with data from viability assays.

DCA effectively depolarizes mitochondria of both normal and cancerous cells

Bonnet *et al.*¹⁹ propose that DCA induces apoptosis through depolarization of mitochondria with a resultant loss of mito-

chondrial transmembrane potential ($\Delta\Psi_m$) and activation of Kv channels. This, it was suggested, results in the release of pro-apoptotic mediators from the mitochondrial matrix leading to cell death. To analyze the effects of DCA on $\Delta\Psi_m$, A549 cells were labeled with TMRM, a rhodamine analog that concentrates in the mitochondrial matrix under an established potential. Cells were treated with increasing concentrations of DCA, MCA, pyruvate, or acetate. Results demonstrated that DCA was the only compound capable of depolarizing cells, with complete loss of $\Delta\Psi_m$ evident after 2 hr at 5 mM DCA (Fig. 3a). Experiments were then extended to

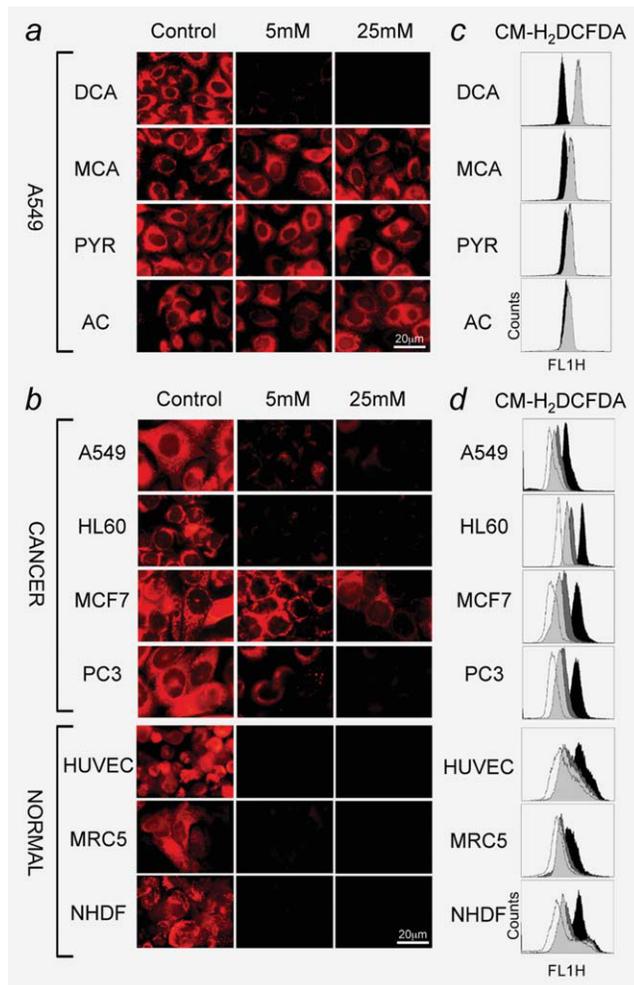


Figure 3. DCA depolarizes mitochondria and increases ROS production in both cancerous and normal cells. (a) A549 cells preincubated with TMRM were exposed to 5 mM or 25 mM DCA, MCA, pyruvate (PYR), or acetate (AC) for 2 hr followed by visualization by fluorescence microscopy. (b) A panel of cell lines preincubated with TMRM was incubated with 5 mM or 25 mM DCA for 2 hr and analyzed as described. (c) A549 cells labeled with CM-H₂DCFDA (10 μ M) were treated with DCA, MCA, pyruvate, or acetate (25 mM) for 2 hr followed by analysis by flow cytometry (FL1) for increases in ROS fluorescence (black histogram = control, gray = treated). (d) The same panel of cancer and normal cell types were treated with increasing concentrations of DCA (0.5, 10 and 20 mM), labeled with CM-H₂DCFDA and analyzed by flow cytometry (open histogram = control, light gray = 0.5 mM, dark gray = 10 mM, black = 20 mM). All results are representative of data from three separate experiments.

include three additional cancer and three normal cell lines (Fig. 3b). Under identical labeling conditions, tumor cell lines were hyperpolarized (greater $\Delta\Psi_m$) relative to normal cells. However, all cell types showed evidence of depolarization with DCA. Complete depolarization was evident in all cell lines except MCF7, which had residual TMRM fluorescence

at 25 mM DCA. These results illustrate that DCA is uniquely capable of depolarizing mitochondria of both cancerous and normal cells.

DCA was also previously shown to increase levels of reactive oxygen species.¹⁹ Therefore, we sought to repeat these experiments using MCA, pyruvate and acetate as reference agents (Fig. 3c). Results demonstrated that DCA-treated HL60 cells had elevated levels of ROS, as determined by CM-H₂DCFDA fluorescence, and that DCA was the most potent ROS generator of the four test compounds. ROS production was also dose dependent and occurred in both tumor and normal cells (Fig. 3d). This increase was partially rotenone sensitive thereby implicating electron transport chain (ETC) complex I as one source and confirming results from Bonnet *et al.*¹⁹ (see Supporting Information Fig. 2). Thus, DCA induces rapid and significant increases in ROS generation irrespective of cellular origin. A further question concerned whether DCA-derived ROS was itself responsible for anti-proliferative activity. To address this, four cell lines (A549, HL60, MCF7 and PC3) were preincubated with the antioxidants L-NAC or ascorbate followed by addition of DCA. Results shown in Supporting Information Figure 3 demonstrate that, in general, both L-NAC and ascorbate enhance DCA activity. This observation suggests that the ROS generated by DCA actually plays a protective role, perhaps by providing negative feedback to the ETC or by directly stimulating glycolysis.²⁷

Furthermore, the mechanism reported previously¹⁹ is reliant on the activity of the redox sensitive potassium channel Kv1.5. It was proposed that DCA-induced efflux of cytochrome c and H₂O₂ from mitochondria activates plasma membrane Kv1.5 resulting in loss of intracellular K⁺ and activation of caspases, contributing to apoptosis induction. We addressed the role of Kv1.5 in DCA activity by attempting to reproduce data demonstrating increases in Kv1.5 mRNA and protein after treatment. Attempts at real-time PCR quantitation of Kv1.5 levels in a panel of cell lines using seven different primers specific to Kv1.5 failed to demonstrate changes in mRNA levels after treatment with a range of DCA concentrations (Supporting Information Fig. 4). Likewise, western blotting of lysates from DCA-treated cell lines using the Kv1.5 antibody described in Bonnet *et al.* failed to demonstrate induction after treatment. Similar results were obtained for two additional Kv1.5 antibodies (Supporting Information Fig. 4).

DCA administered orally is moderately active in A549 xenografts

Bonnet *et al.*¹⁹ report subcutaneous A549 human lung tumor xenograft growth suppression and regressions resulting from DCA administration *via* the drinking water at 0.075 mg/l to athymic nude rats. To validate this observation, we also studied the effect of DCA in athymic nude rats bearing A549-ASC1 human lung tumor xenografts. Tumor growth suppression was evaluated following administration of DCA by twice daily ip dosing at 250 mg/kg/dose given for 21 days (BID \times

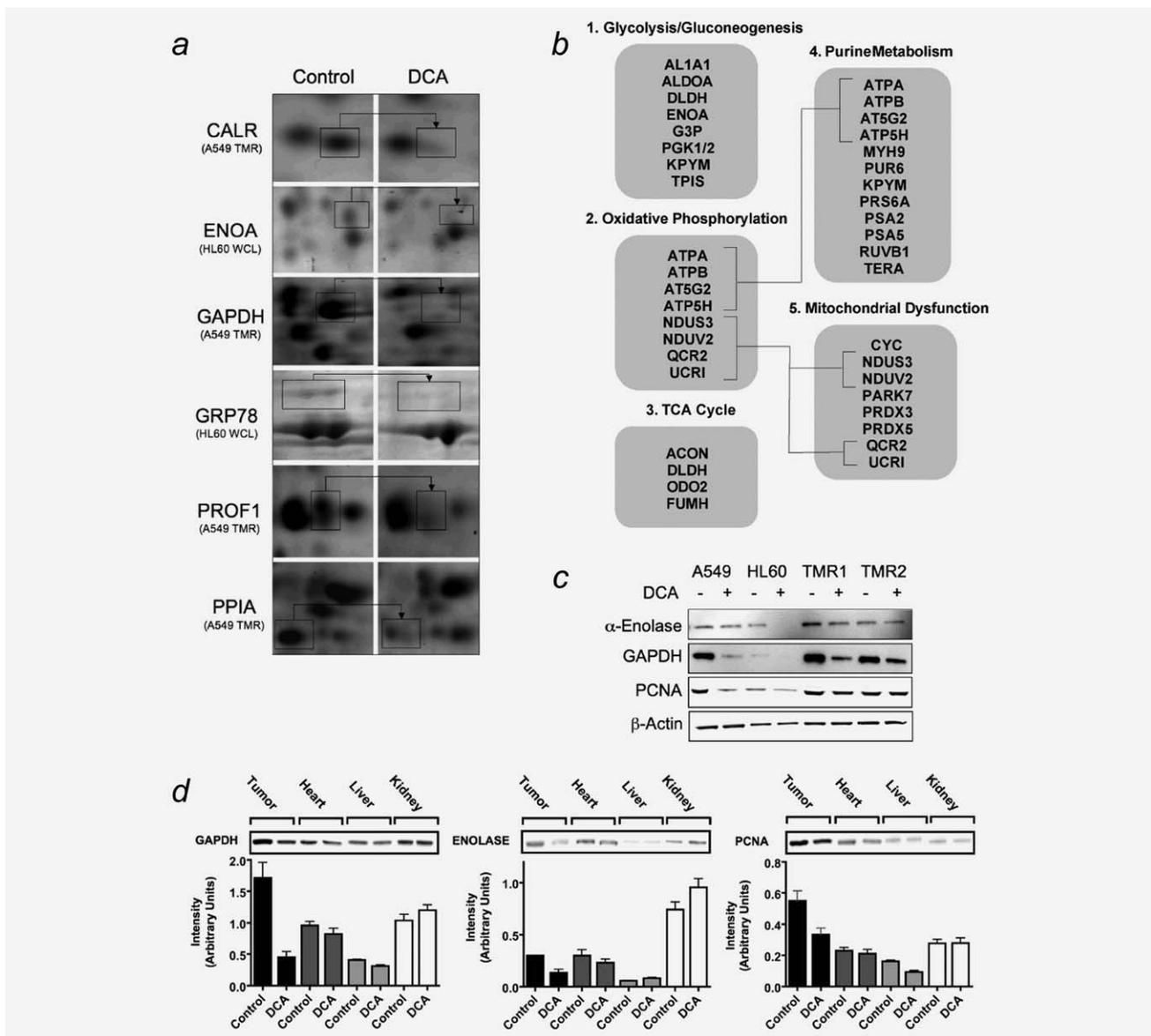


Figure 4. DCA-treated cells exhibit proteomic adaptations associated with mitochondrial dysfunction and growth suppression. (a) Magnified view of selected spot patterns for proteins showing conserved differential expression in DCA treated cell lines and tumors. (b) Highest ranked canonical pathways identified by Ingenuity pathways analysis using all 139 unique protein IDs. (c) Western blotting of lysate from DCA treated A549 and HL60 cells and xenograft tumors using antibodies against α -enolase, GAPDH and PCNA. (d) Western blotting of α -enolase, GAPDH and PCNA expression in tumors and normal tissues isolated from DCA treated A549 xenografts. Results shown in the lower panel are the average densitometric analysis of data from three separate animals. DCA was administered *via* drinking water to athymic nude rats at 0.075 mg/mL.

42) and by drinking water administration at 0.075 mg/mL. We found in two separate experiments tumor growth suppression of A549 ASC-1 (T/C of 53%) when DCA was delivered at 0.075 mg/mL of drinking water, suggesting tumor growth suppression (see Supporting Information Fig. 5). In contrast, parenteral administration was not as effective in decreasing tumor growth. Our experience with A549 ASC-1 xenografts in athymic rats suggests these tumors can spontaneously regress as demonstrated by 4 of 14 tumors in control

rats decreasing after achieving sizes in excess of 500 mg. Therefore, the tumor growth data was not considered beyond the time point when 30% of the control rats were lost from the study. These data confirm that DCA has some activity against A549 xenografts when administered in drinking water.

DCA-treated cells show unique proteomic adaptations

Additional information was sought by performing 2D-PAGE proteomic analysis on DCA treated cell lines and tumor

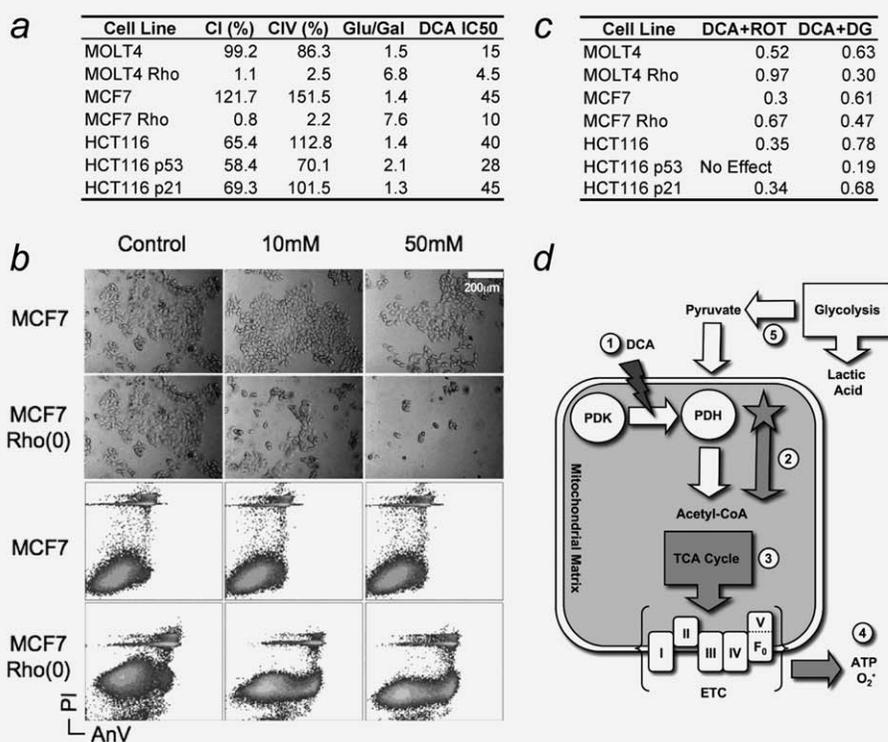


Figure 5. DCA is selectively active against cells with a defective mitochondrial ETC. (a) Metabolic profiling of a panel of cell lines: including ETC complex I/IV activity expressed as a percent of MRC5 activity, relative glucose/galactose growth ratio (Glu/Gal) and DCA cytotoxicity (IC₅₀) values. (b) The effect of 48 hr DCA treatment on MCF7 and MCF7 rho(0) cells in terms of overall cell number (scale bar, top right panel = 200 μm) and apoptosis induction (lower panels). (c) Drug combination studies using DCA + deoxyglucose and DCA + rotenone for the panel of cell lines. Effects of drug combinations were evaluated using the CalcuSyn software as described in Material and Methods. CI values <0.8 indicate synergy, values between 0.8 and 1.2 indicate additivity and values >1.2 indicate antagonism, (–) indicates that rotenone did not have any effect on DCA activity; therefore, a CI could not be calculated. (d) DCA mechanism schematic: as an inhibitor of pyruvate dehydrogenase kinase (PDK) (1), DCA releases pyruvate dehydrogenase (PDH) from negative regulation (2) and promotes entry of pyruvate into the TCA cycle (3). As a consequence, oxygen consumption and ROS production increased (4), whereas glycolysis and lactic acid production are repressed (5). In cancer cells where the Warburg effect is firmly established through defects in the ETC, the effects of DCA are magnified as pyruvate is forced into an inefficient pathway.

tissue. Samples used in the proteomic analysis included DCA-treated whole cell lysate (WCL) and crude mitochondrial preparations (MTO) from A549 and HL60 cells, along with tumor tissue lysate prepared from DCA-treated A549 ASC-1 xenografts (TMR) (see Supporting Information Fig. 5). Because of the relatively mild effect that DCA has on cells and thus the low number of proteins that were modulated, the data from all samples (WCL, MTO and TMR) were combined. A total of 139 unique proteins were differentially modulated by DCA (see Supporting Information Table 1). Figure 4a shows a magnified view of several modulated spots with protein identification. Ingenuity pathways analysis using this combined list of proteins showed that the top five canonical pathways enriched in the dataset were glycolysis/gluconeogenesis, oxidative phosphorylation, citrate metabolism, purine metabolism and mitochondrial dysfunction (Fig. 4b). This data provides support for the importance of metabolic

modulation in DCA activity. Although several proteins involved in cell proliferation (e.g., PCNA) were found to be downregulated, the respective proteomes lacked evidence of apoptosis induction as defined in a previous study of the same cell lines after treatment with a cytotoxic agent.²⁸ Western blot analysis confirmed downregulation of three proteins (α -enolase, GAPDH and PCNA) in cell lines and tumors (Fig. 4c). Western blotting of heart, liver and lung from DCA treated animals confirmed that downregulation of these proteins was tumor specific (Fig. 4d). As additional confirmation, in A549 ASC-1 xenografts where DCA was delivered intraperitoneally, and no tumor regression was observed (Supporting Information Fig. 5), levels of α -enolase, GAPDH and PCNA in tumor tissue were unaffected (Supporting Information Fig. 6). Therefore, proteomic data supports the hypothesis that DCA is a metabolic modulator that negatively impacts cell growth without apoptosis induction.

DCA activity correlates with glycolytic bias and ETC integrity

Given that previous reports have shown that cells with impaired OXPHOS are sensitive to OXPHOS promoting agents,²⁹ we speculated as to whether this was also the case for DCA. Therefore, a panel of cell lines with defined defects in the ETC was assembled comprising: MOLT4 and MCF7 along with their rho(0) counterparts. HCT116 cells were included along with a p53(-/-) variant, chosen based on the recent report that loss of p53 reduces ETC complex IV activity.¹¹ HCT116 p21(-/-) cells were also included as a control because p21 loss has not been shown to be associated with ETC deficiency. An assay for activity of mitochondrial complexes I and IV was then used to confirm molecular defects. Figure 5a shows the complex I/IV activity for each cell line expressed as a percent of MRC5, a “normal” cell line. Results demonstrated that enzymatic activities of both Molt4 and MCF7 cells were similar to MRC5 (Fig. 5a). Conversely, the Molt4 rho(0) and MCF7 rho(0) demonstrated almost total loss in activity for both complexes (0.8–2.5% of MRC5). For the HCT116 cell set, complex I activities were consistent between parental, p53(-/-) and p21(-/-) variants. However, complex IV activity was significantly lower (30%) for p53(-/-) cells, consistent with the reported effects of p53 loss on complex IV expression.¹¹ It should be noted that changes in complex I/IV activity between lines were not a consequence of differences in mitochondrial mass given that staining with 10-N-nonyl-acridine orange (NAO) demonstrated almost identical fluorescence intensities per cell for all lines (see Supporting Information Fig. 7). The panel was also analyzed for growth rates in media containing only glucose or galactose to provide an overall measure of glycolytic bias.³⁰ Cells with defective OXPHOS fail to survive when cultured in galactose-containing medium as metabolism of galactose to glucose-1-phosphate is inefficient. As expected from the complex I/IV results, the rho(0) variants had a higher Glu/Gal ratio than the parental (Fig. 5a), indicating the reduced ability to grow in media devoid of glucose. The ratio for HCT116 p53(-/-) was almost two times higher than parental but substantially lower (3.2–3.6 times) than the rho(0) cells.

After establishing the hierarchy of glycolytic bias, cell lines were evaluated for susceptibility to DCA in cell viability assays (Fig. 5a). Results demonstrated that the rho(0) cells were 3–5 times more sensitive to DCA than parental cells. HCT116 and p21(-/-) variants had identical IC₅₀ values (40–45 mM), whereas p53(-/-) cells were more susceptible (28 mM). Further analysis of MCF7 rho(0) cells demonstrated that increased sensitivity to DCA relative to parental was a consequence of enhanced apoptosis induction (Fig. 5b). Next, drug synergy studies were conducted to evaluate the susceptibility of each line to DCA in combination with the complex I inhibitor, rotenone, or an inhibitor of glycolysis, 2-deoxyglucose (Fig. 5c). If our hypothesis is valid, in cells with ETC defects (reliant on glycolysis), rotenone + DCA should not synergize, whereas in “normal” cells (intact OXPHOS) synergistic activity should be observed. Conversely, deoxyglu-

cose + DCA should synergize in cells with ETC defects, but not in “normal” cells. Results demonstrated that in MOLT4 rho(0) cells DCA failed to synergize with rotenone (CI = 0.97 vs. 0.52 for parental) and for MCF7 rho(0) cells rotenone synergy was significantly lower than that observed in the parental cells (CI = 0.67 vs. 0.3). For both rho(0) cell lines, combining DCA with 2-deoxyglucose showed increased synergy relative to parental (MOLT4 CI = 0.3 vs. 0.63 and MCF7 CI = 0.47 vs. 0.61 for rho(0) vs. parental, respectively). Similarly, 2-deoxyglucose effectively synergized with DCA in p53(-/-) HCT116 cells (CI 0.19), whereas the effect was greatly reduced in parental or p21(-/-) cells (CI of 0.78 and 0.68, respectively). Also, DCA synergized with rotenone in parental and p21(-/-) HCT116 cells but had no effect on the p53(-/-) variant. Overall, these results confirm that DCA has enhanced activity against cells with a defective ETC (see schematic, Fig. 5d).

A logical extension of this work was to determine whether DCA could enhance the activity of agents that interfere with glucose metabolism or directly damage mitochondria (see Supporting Information Fig. 8). Synergy experiments were performed in which A549 cells were treated with DCA in combination with wortmannin or LY 294002, two inhibitors of PI3K, a molecule involved in regulation of glucose metabolism.³¹ Results showed a high degree of synergy for the combination of DCA plus wortmannin (CI = 0.15) or LY 294002 (CI = 0.19). Similarly, DCA enhanced the activity of cisplatin (CI = 0.004) and topotecan (CI = 0.15), two clinically approved agents known to damage the mitochondrial DNA.^{32,33} Therefore, DCA appears to have utility in enhancing the activity of chemotherapeutic agents that target mitochondria or perturb glucose metabolism.

Discussion

The suggestion that dichloroacetate, an inhibitor of PDK, has potential as a cancer therapeutic is highly significant.¹⁹ This structurally simple, orally bioavailable agent has been extensively studied in clinical trials of lactic acidoses and been shown to have relatively modest toxicity.^{15–18} However, the extent to which DCA can perturb cancer cell growth and the underlying mechanism of action remains the subject of debate. Before the inception of this study, a singular mechanistic study of DCA anti-cancer activity suggested a complex pathway where DCA “normalizes” hyperpolarized cancer cell mitochondria, leading to ROS generation, efflux of mitochondrial pro-apoptotic factors and activation of Kv1.5 potassium channels, culminating in programmed cell death.¹⁹ Two subsequent reports focusing on prostate and endometrial cancer cell lines suggested that DCA has some anti-cancer activity.^{20,21} The initial aim of this study was, therefore, an attempt to verify these findings.

The first set of experiments involved establishing IC₅₀ values for DCA across a diverse panel of cell lines. Intriguingly, DCA appeared to be relatively inactive in all lines, with IC₅₀ values in the millimolar range (17–40 mM). Furthermore,

this lack of *in vitro* activity was underscored by results showing that DCA had a similar IC₅₀ to both sodium pyruvate and sodium acetate. These results were shown not to be a consequence of DCA source or viability assay format. Indeed, DCA inhibited PDK at a concentration similar to that reported in the literature. Therefore, it would appear that at pharmacologically relevant doses, DCA had minimal growth inhibitory activity *in vitro*. A retrospective of previous reports appears to confirm these findings given that in Cao *et al.* IC₂₅ values for PC3-neo and PC3-Bcl2 cell lines were 0.5 mM and 1 mM, respectively.²¹ Likewise, in Wong *et al.*, doses of >10 mM DCA were required to inhibit cell viability of endometrial cancer cell lines.²⁰ Importantly, the report of DCA cancer selectivity¹⁹ was questioned by results showing similar IC₅₀ values for DCA in both cancer and normal cells.

Extending the investigation to apoptosis induction yielded similar results. Here, high concentrations (>25 mM) of DCA were required to induce apoptosis in HL60 cells, whereas three other cell lines (A549, MCF7 and PC3) had greater resistance to treatment. This data was supported by immunocytochemistry showing a lack of cytochrome c efflux from mitochondria and an absence of nuclear condensation after treatment. Proteomic analysis of cell lysate and mitochondria from DCA-treated cells also supported this interpretation by showing that changes in markers of cell proliferation were not accompanied by changes in apoptosis related proteins. Similarly, we could not replicate data showing induction of Kv1.5 potassium channel expression after DCA treatment.¹⁹

Nevertheless, DCA was confirmed to have interesting biological properties, including the ability to collapse a mitochondrial membrane potential ($\Delta\Psi_m$) at previously reported doses (0.5–5 mM).¹⁹ Increases in reactive oxygen species with DCA treatment were also confirmed. However, although cancer cell mitochondria appear hyperpolarized relative to normal cells, DCA treatment had equally powerful depolarizing abilities, irrespective of cell type. From a wider standpoint, these data support reports that depolarization of mitochondria does not necessarily result in apoptosis and counters the suggestion that maintenance of a hyperpolarized state is required for cancer cell survival.^{19,34,35}

Having confirmed the ability of DCA to promote OXPHOS, increase ROS production and inhibit cell growth without apoptosis induction, we speculated whether DCA was preferentially active in cells with a defective ETC. To address this, we analyzed a panel of cell lines with known defects in the mitochondrial ETC. Experiments with rho(0) cells appeared to confirm our hypothesis, as they were far

more sensitive to DCA *in vitro*. Results from experiments with p53(–/–) HCT116 cells were also interesting, suggesting that decreased levels of ETC complex IV correlate with increased synergy between DCA and 2-deoxyglucose, whereas conversely decreasing synergy between DCA and rotenone. Therefore, we suggest that in cells with a defective ETC, DCA treatment results in “metabolic crisis” whereby cells are forced to generate ATP using a deficient pathway, resulting in a failure to meet metabolic demands. This overall concept was supported by data showing that *in vitro* DCA synergized with cisplatin and topotecan, two agents known to target mitochondrial DNA.^{32,33}

Our limited exploration of DCA activity *in vivo* using A549 xenografts confirms the experience of Bonnet *et al.*¹⁹ More recently, DCA has also been shown to have moderate *in vivo* activity in models of breast,³⁶ pancreatic,³⁷ and colorectal cancer³⁸ underscoring the need for a comprehensive and standardized exploration of DCA *in vivo* activity (work in progress). That said, a scientific basis for DCA *in vivo* activity remains to be resolved. Although we identify ETC integrity as a factor, it is possible that additional effects of DCA, such as reduced lactate production, decreased intratumoral oxygen or altered acetyl-CoA utilization may account for effects *in vivo*. Likewise, expression of PDK isoenzymes has also been shown to change during the *in vitro* to *in vivo* transition.^{39,40}

In conclusion, a deeper understanding of DCA pharmacology will significantly improve the chances of successful clinical evaluation. Data generated here suggest that *in vitro*, DCA is a relatively innocuous agent that depolarizes mitochondria, increases ROS generation and inhibits cell growth without apoptosis induction. We also confirm that activity of this metabolic modulator is amplified in cells with defects in the mitochondrial ETC. Indeed, we propose that clinical development of DCA may benefit from selecting patients with highly glycolytic tumors. Alternatively, combining DCA with agents that promote ETC inefficiency or inhibit glucose metabolism may have virtue.

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